Thermal Inactivation of Microorganisms

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I. INTRODUCTION

Food preservation is designed to enhance or protect food safety while maintaining the organoleptic attributes of food. Inactivating or inhibiting the growth of undesirable microorganisms is very important for the successful and acceptable preservation of food. While a large number of preservation processes are at the disposition of food processors, the use of adequate heat treatment to destroy pathogenic and spoilage microorganisms is one of the most effective food-preservation processes in use today and has been used for centuries. Heat treatment designed to achieve a specific lethality for foodborne pathogens is a critical control point in food processing and is fundamentally important to assure the shelf life and microbiological safety of thermally processed foods. A key to optimization of the heating step is defining the target pathogen's heat resistance. While overestimating the heat resistance negatively impacts the product quality by altering the organoleptic attributes and nutritional qualities of a food, underestimating increases the likelihood that the contaminating pathogen will persist after heat treatment or cooking. Inadequate heat treatment or undercooking is an important contributing factor in food-poisoning outbreaks (1).

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The extent of heat treatment applied to foods falls into two categories:

1. Low-heat processing or pasteurization (named after Louis Pasteur) refers to the use of relatively mild heat treatment and is widely accepted as an effective means of destroying all non–spore-forming pathogenic microorganisms and significantly reducing the number of natural spoilage microflora, thereby extending the shelf life of pasteurized products. Pasteurization of milk has been performed for decades and is achieved by heating at 145°F (63°C) for 30 minutes or 161°F (72°C) for 15 seconds or equivalent treatments. These time-temperature combinations are sufficient to destroy the most heat-resistant target pathogens in milk, i.e., *Mycobacterium tuberculosis* (organism causing tuberculosis) and *Coxiella burnetti* (Q fever pathogen) and are designated as low-temperature, long-time (LTLT) and high-temperature, short-time (HTST) methods, respectively. The shelf life of pasteurized products depends on the type of food in addition to conditions of pasteurization and subsequent storage (2). Bacterial spores and some heat-resistant enzymes are not destroyed by the pasteurization process and limit the shelf life of the product.
2. High-heat processing or sterilization (in-container) refers to the complete destruction of microorganisms. *Bacillus stearothermophilus* spores, being extremely heat resistant, are often used to evaluate commercial sterilization. This is achieved by application of heat at very high temperatures for a short time to render food free of viable microorganisms that are of public health significance or that are capable of growing in the food at the temperature at which the food is likely to be held (under normal nonrefrigerated storage conditions) during distribution and storage. These products are termed as "commercially sterile." However, this term is not correct because sterility implies the absence of living organisms. But these shelf-stable and microbiologically safe products may contain a low number of dormant bacterial spores. There are two limitations to the above "in-container" sterilization processes: first, products heat and cool at a relatively slow rate; second, final processing temperatures depend on the internal pressure generated (3). While thermal sterilization has been widely used in the food industry for over 200 years because of its proven reliability, ultra–high temperature (UHT) processing, which combines continuous flow thermal processing with aseptic packaging, has been introduced as an alternative sterilization process. Thus, the alternative term "aseptic processing" is also used. By UHT processing, quality of some products is improved because heating and cooling occurs at a faster rate and higher temperatures can be achieved by removing the pressure con-

straints (3). Milk heated to 150°C for 2–3 seconds can be stored at room temperature and has a shelf life of 3 months.

II. INACTIVATION KINETICS PARAMETERS

The higher the initial microbial population in a food, the longer is the processing/heating time at a given temperature required to achieve a specific lethality of microorganisms. Accordingly, the thermal process is designed based on the expected microbial load in the raw product. As such, the heat resistance of bacteria is described by two parameters: D- and z-values. The D-value is the time at a particular temperature necessary to destroy 90% of the viable cells or spores of a specific organism. It is a measure of the death rate or the heat sensitivity of the organism. The z-value is the change in heating temperature needed to change the D-value by 90% (1 log cycle), i.e., z-value provides information on the relative resistance of an organism at different destructive temperatures. D- and z-values are used for designing heat-processing requirements for desirable destruction of microorganisms in a particular food.

Generally, the rate of destruction of bacteria follows first-order kinetics, i.e., when a microbial population is heated at a specific temperature, the cells die at a constant rate. Log number of survivors decline in a linear manner with time (4,5) (Fig. 1). This traditional first-order kinetics model of thermal inactivation forms the basis of calculations used in thermal processing and has served the food industry and regulatory agencies for decades. However, this approach assumes that all of the cells or spores in a population have identical heat resistance, and it is merely the chance of a quantum of heat impacting a heat-sensitive target in a cell or spore that determines the death rate (6). Significant and systematic deviations from classical semi-logarithmic linear declines in the log numbers with time have been frequently observed, even when precise attention is paid to methodology (5,7,8). Such deviations are of two forms: (a) a shoulder or a lag period, i.e., time periods when the bacterial populations remain at the inoculation level; (b) a tailing, i.e., a subpopulation of more resistant bacteria that decline at a slower rate (Fig. 1).

The thermal inactivation data cannot be accounted for by experimental artifacts, and there is presently no satisfactory, unifying explanation for the variability in thermal death kinetics. Hansen and Riemann (9) suggested that the deviations in linear survival curves result from a cell population heterogenous in heat resistance, i.e., due to variability in heat resistance within a population. The "shoulder effect" observed may be attributed to the poor heat transfer through the heating menstruum or may be due to an initial requirement for the bacterial cells to sustain sufficient injury before the first-order inactivation kinetics in the log number of survivors with time. The shoulder also may be attributed to a

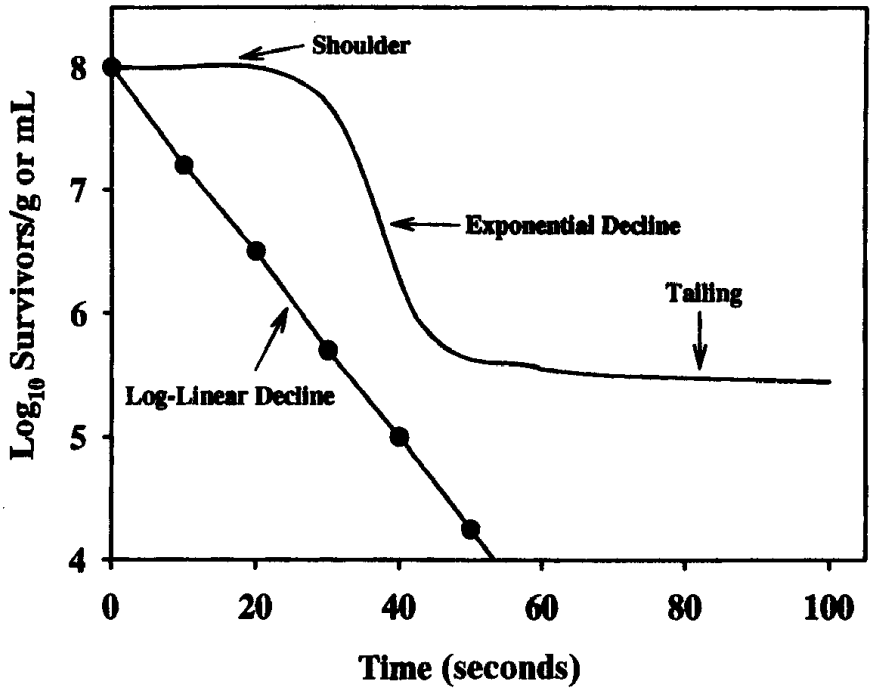

Figure 1 Thermal inactivation of microorganisms. The straight line represents the traditional first-order kinetics of microbial death, i.e., log number of survivors declining in a linear manner with time. The curved line represents microbial death showing a shoulder, a linear decline, and a tailing that are frequently observed in practice.

requirement for more than one damaging event or the need to activate the spores to make them more susceptible to thermal destruction. The "tailing effect" may be due to clumping of small number of cells in the heating menstruum resulting in their protection and increased resistance to the lethal effect of heat (4,9). Cerf (10) proffered two concepts for tailing:

1. A vitalistic mechanism that assumed a variability of heat resistances within a population, i.e., there may be a distribution of heat resistances within a population of cells that constitutes a suspension of apparently identical cells. The relative heat resistance is a genetic trait for an individual cell, and a population of cells presumably formed a normal distribution of cells.
2. A mechanistic concept, which assumed that resistance was the result of physiological and biochemical changes occurring in the cells. Under specific environmental conditions or at different times of its life cycle, an individual cell has varying degrees of heat resistance. A small population of cells is liable to be in a resistant state at the time of heating. Nonlinear survival curves may also be an artifact of the heating or

enumeration technique or nonuniform heat treatment. Also, cells acquire heat resistance as a result of sublethal heating. Such curves are increasingly observed due to the consumer demand for "fresh" and ready-to-eat products such as cook/chill, *sous-vide* foods, etc. Processing of such foodstuffs implies a mild increase in temperature, for which deviations from linearity in the survival curve are frequently observed (11).

Therefore, consideration of non–first-order inactivation kinetics is important in the safe application of milder heat processes or those relying on the combined effects of other factors such as pH, water activity, etc.

Typically, the traditional log-linear death model or a linear regression approach has often been employed to analyze thermal inactivation data despite a poor fit. This has likely resulted in false estimates of heat resistance values for nonlinear survival curves. Accordingly, attempts have been made to explain these deviations (nonlinear survivor curves) by various theories, and several alternate models have been developed to account for this behavior (12–14) and have been excellently reviewed by Whiting (15). Examples of the primary models used to describe survivor curves of microorganisms are given in Table 1. Abrahm et al. (16) hypothesized that the initial shoulder before an exponential decline resulted from the requirement for dormant spores to be activated before being destroyed by heat. Both activation and inactivation were first-order processes, and the first step was the limiting process. Later researchers advanced a population dynamic theory for thermal inactivation of spores. According to this theory, the initial decrease or increase in spore populations was due to a combination of first-order processes for the rapid inactivation of less heat-resistant spores followed by a period of activation of remaining spores to a more heat sensitive state and finally inactivation of remaining spores (17–20). Sapru et al. (21) used these concepts to model dormant spores being either inactivated or activated by heat in ultra–high-temperature sterilization. The temperature dependence of each of the three parameters followed the Arrhenius equation, and the model successfully predicted spore inactivation during variable heating regimes.

III. METHODS FOR DETERMINATION OF HEAT RESISTANCE

A. Traditional Methods

Existing methods for thermal inactivation of microorganisms include TDT (thermal death time) tube, TDT pouch (nylon), TDT can, flask, thermoresistometer, and capillary tube methods (2). These methods suffer from many disadvantages, including time-consuming operations, appreciable heating and cooling lags,

Table 1 Examples of the Primary Models Used to Describe Inactivation Curves of Microorganisms

Survival curves	Mathematical description (equation)
Exponential (first-order kinetic)	$N = Noe^{-kt}$ or $N = No10^{-t/D}$ $K = \frac{2 \cdot 303}{D}$
Linear model[a] (211)	$Y = No \; 0 < tT_L$
Logistic equations	$Y = No - (1/D)(t - T_L) \; t > T_L$
One slope (212)	$\log(M_t/M_o) = 2/[1 + e^{(kt)}]$
Biphasic (two slopes)[b] (212)	$\log(M_t/M_o) = \log(\{2F_1/[1 + \exp(k_1 t)]\} + \{2(1 - F_1)/[1 + \exp(k_2 t)]\})$
Initial lag followed by one-phase killing (one slope)[c] (212)	$\log(M_t/M_o) = \log[1 + \exp(-kt_{1/2})] - \log[1 + \exp\{k(t - t_{1/2})\}]$
Initial lag followed by two-phase killing (two slopes)[d] (211)	$\log(M/M_o) = \log[F_1(1 + \exp(-k_1 t_1))/(1 + \exp(k_1(t - t_1)))] + \log[(1 - F_1)(1 + \exp(-k_2 t_1))/(1 + \exp(k_2(t - t_1)))]$
Activated and dormant species[e] (213)	$n = (n_{oa} + n_{od})\exp\left[\frac{-t}{\theta_i}\right] - n_{od}\exp\left[\frac{-t}{\theta_{ai}}\right]$
Gompertz[f] (202)	$\log(n) = \log(n_o) + a\exp[-\exp(b + ct)] - a\exp[-\exp(b)]$
Vitalistic model[g] (8)	$\log_{10}(\text{viable cell number/ml}) = \alpha + \frac{\omega - \alpha}{1 + \exp\left[\frac{4\sigma(\tau - \log_{10}\text{time})}{\omega - \alpha}\right]}$

[a] T_2 is the lag time prior to initiation of inactivation.

[b] Where F_1 was the fraction of population in the major group, k_1 was the inactivation rate parameter for the major population, and k_2 was the inactivation rate parameter for the subpopulation.

[c] Where $t_{1/2}$ was the time for $M = (M_o/2)$, a measure of the lag time.

[d] Where t_1 was the lag period, F_1 the fraction of cells in the major population, and k_1 and k_2 the respective rate parameters ($D = 2.3/k$). When the subpopulation did not exist, the fraction of cells in the subpopulation was set to an insignificantly low value. If the shoulder was not present, t_1 was set to 0.0 and the model became a nearly straight line.

[e] n_{oa}, n_{od} are initial population sizes of activated spores and dormant spores, respectively; θ_i, time constant for inactivation; θ_{ai}, combined time constant for inactivation and activation.

[f] a, b, and c are fit parameters.

[g] Where α is the upper asymptote (log cfu/mL), ω is the lower asymptote (log cfu/mL), σ is the maximum slope of the death curve, log cfu/mL against log time, and τ is the log time at which maximum slope is reached.

splashing of contents, flocculation, high initial cost, and hazards of contamination during subculturing (2). Additionally, using traditional methods, the published literature on the heat resistance of certain organisms, such as *Listeria monocytogenes*, is conflicting. Using the holding technique of pasteurization in screw-capped test tubes placed in a water bath (61.7°C, 35 min), survival occurred when *L. monocytogenes* population levels exceeded 3 log cfu/mL (22). In contrast,

using sealed borosilicate glass tubes, *L. monocytogenes* was unable to survive pasteurization (23). However, later investigation by Donnelly et al. (24) proved how important the methodology is in determining thermal inactivation. The authors compared the heat resistance of *L. monocytogenes* using the sealed tube and test tube methods and concluded that survival of the organism at pasteurization temperatures depended on the method used to inactivate cells and is not a biological phenomenon. Using the sealed-tube method of inactivation, *L. monocytogenes* was easily inactivated at pasteurization temperatures. However, when an identical cell population was heated using the test tube inactivation method, survival of the pathogen was observed, regardless of the heating temperature (62, 72, 82, or 92°C). The authors stated that the condensate and splashed cells could collect in the cap of the test tube above the level of the water bath and drip back into the heating menstruum. Thus, the tubes will have various levels of survivors depending on the amount of condensation in the cap. Also, the authors indicated that cells could coat the walls of the test tubes above the level of water bath; the only cell population exposed to the inactivation temperatures would be that which is below the level of water in the water bath.

B. Submerged-Coil Heating Apparatus

This is a novel and convenient tool for investigating the thermal inactivation kinetics of foodborne spoilage and pathogenic microorganisms (Fig. 2). It is com-

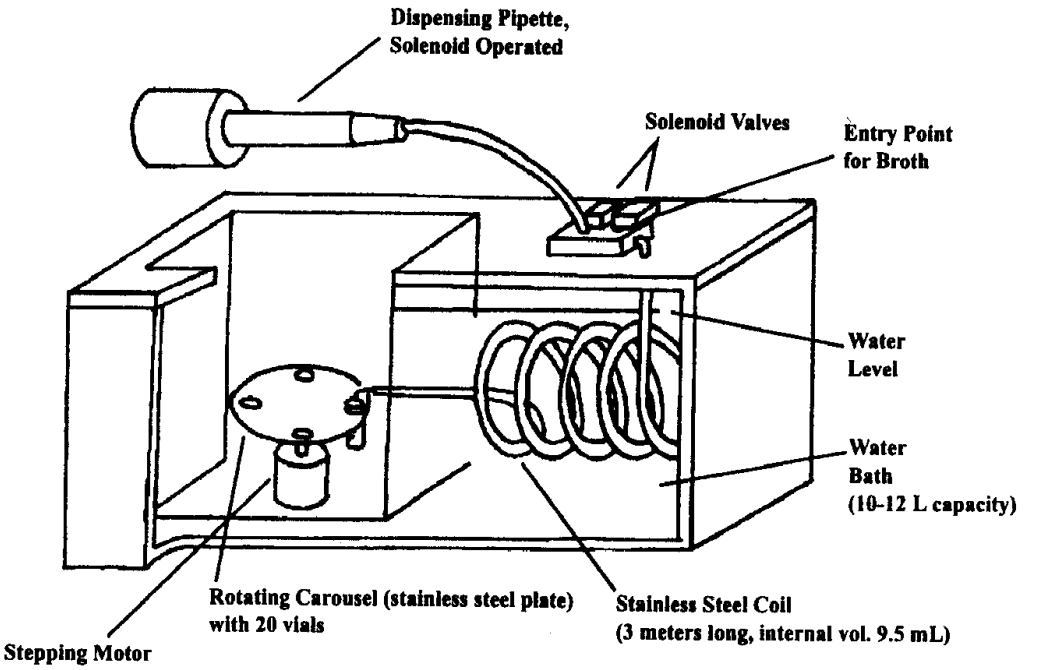

Figure 2 Submerged-coil heating apparatus.

prised of a stainless steel coil, fully submerged in a thermostatically controlled water bath, which allows microbial suspensions to be rapidly heated between 20 and 90°C. Since the equilibrium or come-up time of the samples is negligible, no bacterial inactivation occurs during this period. An electronically controlled displacement mechanism controls the sampling intervals and the number of samples to be dispensed. This makes the equipment fully automated. Various sampling times can be set at intervals ranging from 6 to 999 seconds.

C. Operation of the Apparatus

Bolus microbial suspensions (≤9.5 mL) are loaded rapidly (<1 s) via a solenoid valve system using a disposable syringe into the preheated coil. The come-up time is <1 second. Thereafter, several measured quantities of sterile water are introduced into the charging end of the tube, displacing the heated broth through the coil and discharging sample at the terminal end. These are collected in a series of vials carried on the carousel. The sample delivery needle is automatically flushed twice with hot culture prior to sample displacement to ensure no carry-over of the previous sample. The results obtained using this apparatus were highly reproducible. Thus, the heat resistance of microorganisms in a variety of liquid foods can be quantified accurately. The only disadvantage is that the apparatus cannot be used for assessing the heat resistance of microorganisms in solid foods.

IV. MECHANISM OF HEAT INACTIVATION OR BACTERICIDAL OR SPORICIDAL ACTIVITY

The resistance of bacterial cells and spores to heat has been a focus of study for decades. The literature on heat-resistance mechanisms includes excellent reviews (25–34). Heat resistance in spore-formers has been correlated with DNA content (35), dipicolinic acid (DPA), and calcium chelates with DNA (36), mineralization (37), dehydration (38), as well as thermal adaptation (39). Gerhardt and Marquis (26) reported that heat resistance of spores is attributed primarily to three physiochemical determinants that impact the protoplast: thermal adaptation, mineralization, and dehydration.

Thermal adaptation has been assumed to be an inherent or intrinsic molecular component that is genetically determined. In general, the spores of thermophilic species are inherently more resistant that those of mesophiles, which, in turn, are more resistant than spores of psychrophilic species (40). Furthermore, spores of a particular species or strain produced at higher temperature are more heat resistant than those prepared at optimum or low temperatures (26,39,41,42), and so there appears to be an extrinsic element imposed on the genetic element. Sporulation at higher temperatures inducing additional heat resistance may be

attributed to the reduced water content in spores produced at higher temperatures (38). High sporulation temperatures have been argued to increase the mineralization of spores (39). The correlation between sporulation temperature and heat resistance has been investigated (43,44). Similarly, bacterial cells grown at higher temperatures are known to be more heat-resistant than those grown at lower temperatures (44,46).

Mineralization of the protoplast can have a significant effect on spore heat resistance; the heat resistance of spores is directly related to the level and type of divalent cation. In general, the higher the level of divalent cation, the higher the heat resistance of spores. However, the effect appears to be lower at higher temperatures (47). Setlow (48) reported on the analysis of spores of several microbial species from which all mineral ions were removed by acid titration and then restored by backtitration with a mineral hydrooxide. This work gave the following order of increasing spore heat resistance with different cations: $H^+ < Na^+ < K^+ < Mg^{2+} < Mn^{2+} < Ca^{2+}$ = untreated. Spores of *Clostridium botulinum* containing an increased iron content in the sporulation medium were more sensitive to heat compared to the spores with normal iron levels or spores with increased zinc or manganese. Spores formed with added manganese or zinc were better able to repair heat-induced injuries than spores with added Fe or Cu (49). While mineralization of spores in the later stages of sporulation is associated with an uptake of DPA, the latter is not thought to be necessary for attaining heat resistance, but there is growing evidence that it may have a role in retaining the resistance (26). Beaman and Gerhardt (38) reported that the increases in mineralization of spores is accompanied by reductions in protoplast water content, and at least a part of the increased heat resistance associated with mineralization is due to dehydration.

Dehydration of the protoplast restricts the mobility of heat-labile components of the spore core, i.e., vital macromolecules such as proteins, RNA, and DNA, and renders them less sensitive to irreversible thermal denaturation or even prevents denaturation (27). Gerhardt and Marquis (26) suggested that the dehydration of the protoplast is the only determinant necessary for the heat resistance of spores. Once protoplast dehydration is obtained or the resistant state is attained, its maintenance is clearly a function of an intact peptidoglycan cortex, but not a coat or exosporium (26). It must be noted that although dehydration is the only property necessary and sufficient in itself to impart heat resistance, it is enhanced by mineralization (especially by calcification) and thermal adaptation.

Spores contain a number of unique core-located, acid-soluble, basic proteins. *Bacillus subtilis* spores genetically deficient for synthesis of the α and β small acid-soluble spore proteins (α/β-type SASP) as a result of mutations in genes coding for major proteins of this type have been found to be more sensitive to heat than SASP sufficient spores (50–52). The D-values of *B. subtilis* spores lacking α/β-type SASP (termed as α-β spores) were 10- to 20-fold lower than

those of wild-type spores (53). Inactivation of *B. subtilis* α-β spores is associated with induction of high level of mutations and significant DNA single-strand breakage compared to wild-type spores, in which killing by heat is associated with no significant mutagenesis and many fewer single-strand breaks (51,53). The precise cause of the DNA damage and mutagenesis by heat in α-β spores is not known, but Fairhead et al. (53) suggested that the heat treatment causes DNA depurination followed by strand cleavage. The authors demonstrated that the binding of α/β-type SASP to DNA in vitro reduces the rate of DNA depurination at least 20-fold. Thus, depurination is an attractive explanation for the DNA damage caused by heat treatment of α-β spores. Later, Setlow and Setlow (54) provided direct evidence for this process in vivo by analyzing the level of abasic sites in DNA from heat-inactivated α-β spores directly. Findings of this study suggested that a major mechanism responsible for the heat killing of α-β- (but not wild-type) spores is DNA depurination followed by strand breakage at the resultant abasic site.

Several studies have documented correlations between bacterial membrane fatty acid composition and heat resistance of the organism (55–58). Hansen and Skadhange (59) reported that the reduced heat resistance of cells grown at low temperatures may be due to an increase in the concentration of unsaturated fatty acids in the cytoplasmic membrane, which increases membrane fluidity and reduces viscosity, thereby decreasing thermotolerance. *Vibrio parahaemolyticus* grown at different temperatures and at different sodium chloride concentrations showed alterations in membrane fatty acid profile that correlated with an altered response to subsequent heat treatment (55). Juneja et al. (58) demonstrated an altered heat resistance along with altered membrane fatty acid profiles in *L. monocytogenes* grown in different acidic environments at different temperatures.

Dry heat lethal efficacy is less than that of wet heat treatment and requires higher temperatures and longer times to yield the same killing effect. Ernest (60) suggested that dry heat causes death by the destructive oxidation of cell components. Hashimoto et al. (61) proposed that the heat killing of spores is primarily due to physical and chemical alterations, which interfere with the absorption of water into the core during germination.

V. TARGETS OF HEAT DAMAGE

Heat is believed to be uniformly distributed in a cell, resulting in damage to only the most sensitive molecules within that cell (62). Potential targets of heat damage have been implicated with associations to various pathogen viabilities. These include proteins and enzymes, cellular membranes, as well as nucleic acids (63). Deficiency in DNA repair appears to cause spores to be more heat sensitive (64).

Hanlin et al. (64) suggest that heat-induced DNA damage, if repairable, may not result in inactivation of the spores, and the death of spores with damaged DNA may be due to the inability of spores to germinate and outgrow. Melting of spore DNA may occur at temperatures above 90°C and is reflected in differential calorimetric scans of spores as an endothermic peak at 90–91°C (65,66). In contrast, spore DNA is protected by small acid soluble proteins that bind tightly and specifically to the A form of DNA reducing the rate of depurination in vitro by at least 20-fold (67).

An increasing amount of evidence suggests that ribosome damage and degradation is the cause of cell death following thermal stress (68,69). Ribosome denaturation occurs in the same temperature region as thermal inactivation. Numerous investigators have used differential scanning calorimetry (DSC) to examine thermal transitions as indicators of potential sites of cellular injury (66,70). DSC has proven to be an effective technique in measuring changes in protein denaturation temperatures with corresponding changes in denaturation enthalpy (71). It was suggested that half the enthalpy of ribosome denaturation is associated with protein denaturation and/or disruption of higher-order interaction (72). Anderson et al. (70) reported that measurement of viability loss in the differential scanning calorimeter gave good correlation between cell death and the first major thermogram peak; the peaks observed in the thermogram of the ribosome cell fraction correspond to the major peak in whole cells. Allwood and Russell (73) observed a direct correlation between loss of RNA and heat-induced loss of viability of *Staphylococcus aureus* at temperatures up to 50°C. Magnesium is known to have a stabilizing effect on ribosomes. In a study involving mild heating of *S. aureus*, Hoa et al. (74) reported that heating results in membrane damage, leading to the loss of Mg^{2+} ions and destabilization of the ribosomes. In fact, depletion of Mg^{2+} leads to 70S ribosome dissociation into 30S and 50S subunits, ribonuclease activation, and finally destruction of 30S subunits (75–77). Earlier studies including a number of bacterial species showed that the 30S ribosomal subunit is specifically destroyed during heat treatment, while the 50S ribosomal subunit appears to be stable, and that 16S rRNA is the prime target of degradation in the heat-injured cells while 23S rRNA appears to be unaffected (78). Miller and Ordal (79) examined the rRNA profiles of cells at various times during heat injury at 47°C. The degradation of rRNA and ribosomal subunits occurs differently during heat injury; the 16S and 30S subunits are affected more readily following heating for 5 minutes, and the 23S and 50S subunits are degraded more slowly, disappearing after 30 minutes of heat treatment.

Stephens and Jones (80) proposed that the protection of the 30S subunit is a critical mechanism for increased thermotolerance. In their study, the osmotic and heat shock–induced increased thermotolerance response of *L. monocytogenes* was concurrent with increased thermal stability of the 30S ribosomal subunit, as measured by differential scanning calorimetry. The authors proposed that the

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stabilization of the subunits occurred through cellular dehydration leading to an increase in the internal solute concentration, including Mg^{2+} ions, which may contribute to tighter coupled particles of the 30S subunits. Tolker-Nielsen and Molin (81) reported that heat lethality of *S.* Typhimurium coincides with a significant reduction in the cellular content of 16S ribosomal RNA, thereby suggesting that the degradation of ribosomal RNA is a direct cause of cell death. This conclusion is based on the findings of carbon-starved and magnesium-supplemented cells, which survive heat treatment much better and which also maintain stable levels of ribosomal RNA.

Proteins and enzymes are also considered to be potential sites responsible for heat lethality. It has been postulated that water that is in close contact with the proteins inside the cell could be a factor determining the cell's inactivation. As the cell is heated, water molecules begin to vibrate, and this vibration causes the disulfide and hydrogen bonds in the surrounding proteins to weaken and break, altering the final three-dimensional configuration and possibly preventing the protein from functioning (82). The crucial protein that is the rate-limiting, primary target in heat killing is unknown, but the current belief is that membrane proteins may be denatured by heat initially because of peripheral locations followed by the denaturation of crucial proteins within ribosomes (65). There is evidence that catalase and superoxide dismutase (SOD) may be sensitive to heating. These enzymes detoxify oxygen radicals like superoxide and hydrogen peroxide, which form spontaneously in the presence of oxygen and, if undisturbed, can result in death of the cells as a result of lipid peroxidation and membrane damage (83). Amin and Olson (84) found that staphylococcal catalase activity decreased 10- to 20-fold faster at 54.4°C than at 37.8°C, and Dallmier and Martin (85) found that the specific activity of SOD produced by *L. monocytogenes* decreased quickly when heated to 55°C. In a study on the heat stability of *Bacillus cereus* enzymes within spores and in extracts, Warth (86) observed a range of sensitivities for spore enzymes and concluded that the enzymes in extracts of spores were inactivated at temperatures ranging from 24 to 46°C lower than those needed to inactivate the same enzymes within the intact spores. Membrane-bound ATPase has been associated with heat resistance/sensitivity of microorganisms. Coote et al. (87) suggested that ATPases are essential for the basal heat resistance of the cell to cope with elevated temperatures. Nevertheless, thermotolerance induced by sublethal heating is a mechanism independent of ATPase activity.

Flowers and Adams (88) suggested that the cell membrane is the site of thermal injury of spores subjected to mild or sublethal heating; membrane damage consequently increases sensitivity to environmental stresses. When spores are lethally heated, damage to the membrane permeability barrier results in the release of intracellular constituents and there is a temperature-dependent progressive loss of calcium and DPA (89–91). The death of spores proceeds faster than the release of DPA (65). When vegetative cells are heated, there is a rapid efflux

of ions, amino acids, and low molecular weight nucleic acid components, thereby suggesting that interference with the semipermeability of membranes is a common consequence of heating (5).

A. Heat-Shock Response

While thermal processing guidelines are generally adequate for destruction of pathogens in foods, there may be conditions when the microorganism could become more heat resistant. Such conditions include environmental stresses occurring prior to cooking such as sublethal heat treatment, also known as heat-shocking conditions. Heat shock triggers a physiological response that leads to the synthesis of a specific set of proteins known as heat-shock proteins (HSPs) (92,93). HSPs may enhance the survival of pathogens in foods during exposure to high temperatures. Microbial cells in which HSPs are synthesized acquire enhanced thermal tolerance to a second heat challenge that would normally be lethal to them. Generally speaking, heat-shocked cells have to be heated twice as long as non–heat-shocked cells to achieve the same extent of lethality (94). Heat-shock response and induced thermotolerance has been reported in a wide range of microorganisms including *Escherichia coli* (95), *Salmonella* Typhimurium (45,96), *Salmonella* Thompson (46), *Salmonella* Enteritidis phage type 4 (97), *L. monocytogenes* Scott A (98,99), and *E. coli* O157:H7 (11,100,101). An increase in heat resistance of spores following heat shock has also been reported in spore-forming organisms such as *B. stearothermophilus* (102,103), *C. botulinum* (104), and *Clostridium sporogenes* (105). Although the scientific literature provides some evidence regarding the cause-and-effect relationship between the synthesis of HSPs and the induced thermotolerance response, this evidence is largely indirect and insufficient. Researchers have suggested that HSPs are not necessarily the major contributory agents in the development of thermotolerance but are required for recovery from heat stress (106–108). Their apparent role is to protect the cells against heat damage and to help the cells to return to their normal physiological state following the stressful event. Schlesinger (109) and Sanchez and Lindquist (110) suggested that the role of HSPs in thermotolerance may be to act as chaperones to remove denatured proteins. The primary function of classical chaperones, such as the *E. coli* DnaK (HSP 70) and its co-chaperones, DnaJ and GrpE, and GroEL (HSP60) and its chaperone, GroES, is to bind to and stabilize polypeptides already present in cells, modulate protein-folding pathways to prevent misfolding and aggregation of proteins, and promote refolding and proper assembly (111). Some *E. coli* HSPs are able to proteolyze irreversibly damaged polypeptides and assist in nucleic acid synthesis, cell division, and motility by promoting synthesis of a flagellum (112). In *E. coli*, regulation of stress response has been studied in greater detail through the transcriptional control of alternate sigma factors encoded by *rpoS* and *rpoH* in response to general stress and heat, respectively (113,114).

VI. FACTORS AFFECTING HEAT RESISTANCE

An appropriate heat treatment designed to achieve a specified lethality of microorganisms is influenced by many factors, some of which can be attributed to the inherent resistance of microorganisms, while others are due to environmental influences. Examples of inherent resistance include the differences among species and the different strains or isolates of bacteria (assessed individually or as a mixture) and the differences between spores and vegetative cells. Environmental factors include those affecting the microorganisms during growth and formation of cells or spores (e.g., stage of growth, growth temperature, growth medium, previous exposure to stress) and those active during the heating of bacterial suspension, such as the composition of the heating menstruum (amount of carbohydrate, proteins, lipids, solutes, etc.), water activity (a_w), pH, added preservatives, method of heating, and methodology used for recovery of survivors. This chapter deals with the most significant research on the factors affecting the heat resistance of foodborne pathogens.

VII. HEAT RESISTANCE OF VEGETATIVE FOODBORNE PATHOGENS

The heat resistance of foodborne pathogens has been studied in different substrates. Comparing the heat resistance of some pathogens, such as *L. monocytogenes*, *Salmonella* spp., and *E. coli* O157:H7, it appears that *L. monocytogenes* is relatively more heat resistant (Table 2). However, it is practically feasible to inactivate this pathogen by the type of mild heat treatment given to minimally processed foods without negatively impacting the product quality.

The pH of the heating menstruum is recognized as one of the most im-

Table 2 Heat Resistance of Three Foodborne Pathogens in Meat Expressed as D-Values in Minutes

Menstruum/temperature	*E. coli* O157:H7	*Salmonella* spp.	*L. monocytogenes*
Beef/60°C	3.17[a]	5.48[c]	8.32[d]
Beef/57.2°C	5.3[g]; 4.5[f]	5.4[e]	5.8[h]
Beef/62.8°C	0.5[g]; 0,4[f]	0.7[e]	1.2[h]
Chicken/60°C	1.63[a]	5.20[c]	5.29[d]
Turkey/60°C	1.89[b]	4.82[c]	—
Pork/60°C	2.01[b]	6.65[c]	—

[a] (214); [b] (215); [c] (118); [d] (216); [e] (217); [f] (119); [g] (218); [h] (219).

portant factors influencing the heat resistance of bacteria. Microorganisms usually have their maximum heat resistance at pH values close to neutrality; a decrease in the pH of the heating medium usually results in decreased D-value. Reichart (115) provided a theoretical interpretation of the effect of pH on microbial heat destruction and described a linear relationship between pH and the logarithm of the D-values for *E. coli*. The author stated that the logarithm of the heat-destruction rate increases linearly in the acid and alkaline range and has a minimum at the optimum pH for growth. High pH interacts synergistically with high temperature to destroy gram-negative foodborne pathogens (116). Growth temperature history and pH, including the type of acidulant used to adjust the pH, influence the heat resistance of *L. monocytogenes* strain Scott A (58). D-values significantly decreased ($p < 0.05$) with increased growth temperature when the pH of the growth medium was 5.4; the values significantly increased ($p < 0.05$) with increased temperature at pH 7, regardless of acid identity. At pH 5.4 adjusted with lactic acid, D-values ranged from 1.30 minute for 10°C-grown cells to 1.14 minute for 37°C-grown cells. At pH 5.4 adjusted with acetic acid, *L. monocytogenes* failed to grow at 10°C; the D-values were 1.32 minute and 1.22 minute when the organism was grown at 19 and 37°C, respectively. At pH 7 adjusted with lactic acid, D-values were 0.95, 1.12, and 1.28 minute for cells grown at 10, 19, and 37°C, respectively; the values ranged from 0.83 minute for 10°C-grown cells to 1.11 minute for cells grown at 37°C and pH adjusted with acetic acid. Alternatively, if conditions can be found that produce a more susceptible cell, they can be exploited to enhance inactivation (58).

The protective effect of fatty materials in the heating medium on the heat resistance of microorganisms is well documented (117). Theories behind increased heat resistance in foods with higher fat contents relate to reduced water activity or poor heat penetration (lower heat conductivities) in the fat portion (118). Doyle and Schoeni (119) reported a D-value at 60°C of 0.75 minute for *E. coli* O157:H7 strain 932 in ground beef containing 17–20% fat. Ahmed et al. (120) reported that D-values of *E. coli* O157:H7 in ground beef heated at 60°C ranged from 0.45 (beef, 7% fat) to 0.47 (beef, 20% fat) minute; the values ranged from 0.38 (chicken, 3% fat) to 0.55 (chicken, 11% fat) minute in chicken. These authors reported that the D-value of *E. coli* O157:H7 in ground turkey and pork sausage heated at 55°C in thermal death time tubes ranged from 6.37 (turkey, 3% fat) to 9.69 (turkey, 11% fat) minutes and 6.37 (pork sausage, 7% fat) to 11.28 (pork sausage, 30% fat) minutes; the values at 60°C ranged from 0.55 (turkey, 3% fat) to 0.58 (turkey, 11% fat) minute and 0.37 (pork sausage, 7% fat) to 0.55 (pork sausage, 30% fat) minute. In another study, Ahmed and Conner (121) reported D-values at 55°C for *E. coli* O157:H7 ranging from 12.5 (turkey, 3% fat) to 11.0 (turkey, 11% fat) minutes; the value at 60°C was 0.9 minute regardless of the percentage of fat in turkey. Ground beef contaminated

with *S.* Typhimurium DT 104 heated to an internal temperature of 58°C for 53.5 (7% fat) or 208.1 minutes (24% fat) results in 7-D process for the pathogen; the heating time at 65°C to achieve the same level of reduction is 7.1 and 20.1 minutes, respectively (118). The authors reported that the pathogen does not possess any unique characteristics that would predispose it to survival during thermal processing. Vacuum-packaged pasteurized salmon fillets (10.56–17.2%, w/w, fat) had one to four times higher D-values for *L. monocytogenes* than the lower fat (0.6–0.8%, w/w, fat) cod fillets (122). However, Donnelly and Briggs (123) reported little difference in D-values for *L. monocytogenes* in skim milk, 11% milk solids, and whole milk when fat was studied as a single factor affecting heat resistance. In another study, differences were observed in D-values for *L. monocytogenes* in sheep, cow, and goat milks (124). Sheep milk fat added to cow and sheep skim milks resulted in higher D-values compared to the values obtained from the addition of cow milkfat. Obviously, it is not solely fat content but also the fatty acid composition that affects heat resistance.

Various solutes in the heating medium exert different effects on the heat resistance of microorganisms, depending upon the nature of the solutes and their concentration. The effects of solutes on thermal resistance have mainly been examined by determining the relationships between thermal resistance and either solute concentration or water activity of the heating menstruum. *S. aureus* exhibited increased heat resistance when heated in different substrates with reduced water activity (125). In a study by Reichart and Mohacsi-Farkas (126), when heat destruction of seven foodborne microorganisms as a function of temperature, pH, redox potential, and water activity was assessed in synthetic heating media, the heat destruction increased with increasing water activity and decreasing pH. While an increased heat resistance is observed when the a_w of the heating menstruum is lowered, there appears to be no direct correlation between a given a_w level and heat resistance. For example, sucrose protected salmonellae from heat destruction far more efficiently than glycerol at given a_w levels down to 0.87 (127). Tuncan and Martin (128) suggested that the effect of salts on thermal inactivation of microorganisms is mainly related to reduced water activity and increased osmotic pressure of the heating menstruum. Sodium chloride at different concentrations (3, 5, and 9% w/v) protected *S. aureus* from heat injury, with the highest concentration affording the maximum protection (129). The authors explained that sodium chloride may be involved in stabilizing membrane protein structures such as nucleic acids and nucleotides as well as increasing the melting temperatures of membrane phospholipids. Thus, the damage to the cell membrane and leakage of cell components from the cytoplasm is prevented. In another study, 4 and 8% sodium chloride protected *S. aureus* cells from heat injury at pH 7.0, while at pH 6.5 a concentration of 8% gave protection (130). The D-values at 65.6°C for *S.* Typhimurium ranged from 0.29 to 40.2 in sucrose solutions with a_w ranging from 0.98 to 0.83 (131). For *L. monocytogenes*, the D-values at 65.6°C

shifted from 0.36 to 3.8 minutes in sucrose solutions with a_w ranging from 0.98 to 0.90. The values ranged from 1.2 to 3.2 minutes, while the a_w of the chocolate syrups ranged from 0.75 to 0.84 (131). The addition of small amounts of water to low-a_w foods has been noted to greatly lower the heat resistance of microorganisms. Researchers have reported that the survival of salmonellae in dry egg white over the range 50–70°C decreased rapidly when the moisture level of the product was increased from 3 to 12%, but the a_w levels of these products were not reported (132). Similarly, Van Cauwenberge et al. (133) found that heating at 47°C was more effective for a corn flour product with a moisture level of 15% than for one with a 10% moisture level in reducing the bacterial population from 10^5 to 10^3 cells per g. Archer et al. (134) reported that heat resistance of *S.* Weltevreden increased as the initial a_w of the flour prior to heating decreased and that the relative humidity of the atmosphere during preconditioning did not have a significant effect.

Both an increase in the number of viable cells capable of producing colonies and an increase in the estimated D-value are observed under optimum recovery conditions. Temperatures below the optimum for growth may enhance repair of heat damage (135). Higher recoveries of sublethally heat-damaged cells in anaerobic conditions or when the recovery media is supplemented with oxygen scavengers or reducing agents have been observed in studies with *E. coli* O157:H7 (100,117,136–139), *L. monocytogenes* (140–146), *S.* Enteritidis (147), and *S. aureus* (148). Xavier and Ingham (147) measured heat resistance of *S.* Enteritidis and reported D-values at 52°C of 11.3 and 5.4 minutes and at 58°C of 1.5 and 1.0 minutes when heated cells were recovered under anaerobic and aerobic conditions, respectively. Similarly, for *L. monocytogenes*, the D-value at 55°C was increased from 8.9 to 12.0 minutes when prereduced TSAYE plates were incubated anaerobically rather than aerobically (142). The increased resistance of *L. monocytogenes* under anaerobic conditions may explain why the pathogen appeared to survive pasteurization at 62.8°C for 15 minutes when recovered anaerobically (141) and why minimal heat treatment had only a marginal effect on the pathogen inoculated into chicken gravy (149) and *sous-vide* chicken breast (150). The D-values of *E. coli* O157:H7 at 55°C were reported to be 8.0, 11.1, and 18.3 minutes following recovery on aerobic TSA plates, on anaerobic TSA plates, and in anaerobic roll tubes, respectively (100,137). Interestingly, George et al. (139) observed that the measured heat resistance of cells was greatly influenced by oxygen concentration. The heat resistance of *E. coli* O157:H7, *S.* Enteritidis, and *L. monocytogenes* was up to eightfold greater when they were grown, heated, and recovered anaerobically rather than aerobically. The authors reported that the time at 59°C for a 6-D reduction of *E. coli* O157:H7 was 19–24 minutes; this was reduced to 5–17 minutes when 0.5–1% oxygen was included and to 3 minutes when 2–40% oxygen was used. Further, Bromberg et al. (138) reported that oxygen-sensitive cells were only sublethally heat damaged and regained their

ability to grow in the presence of oxygen when allowed a recovery period in anaerobic conditions. George and Peck (151) differentiated between the effects of oxygen and that of high redox potential and reported that sublethally heat-damaged cells regained their ability to grow in media of high redox potential at a similar rate whether the redox potential was increased by the addition of potassium ferricyanide, 2,6-dichloroindophenol, or oxygen.

Addition of various concentrations of the bacteriocin nisin renders the bacteria sensitive to the lethal effect of heat, thereby enhancing the effectiveness of the thermal processes during mild heat treatments. Boziaris et al. (152) reported a reduction of required pasteurization time of up to 35% for *S.* Enteritidis when the heating menstrua (nutrient media, liquid whole egg, and egg white) was supplemented with 500–2500 IU/mL of nisin. The pathogen was most sensitive in egg white that had an alkaline pH and contained no fat. In a study by Budu-Amoako et al. (153), when nisin was added at a level of 25 mg/kg of can contents to the brine surrounding the lobster, in combination with a heat process giving internal temperatures of 60°C for 5 minutes and 65°C for 2 minutes, a 3–5 log reduction of *L. monocytogenes* was observed compared to heat or nisin alone, which resulted in 1–3 log reductions of the pathogen.

It has been well documented that bacterial cells/population in stationary phase or those that have undergone some sublethal stress undergo physiological changes that make them more resistant to subsequent heat treatment or any other potentially stressful condition (154). For example, sublethal heat stress renders an organism more resistant to subsequent heat treatment that would otherwise be lethal. Cross protections in which exposure to one stress alters resistance to another can also occur. For example, acid pH exposure or osmotic shock can induce stress responses including resistance to thermal stress (155,156). Adaptation of *L. monocytogenes* to starvation, ethanol, hydrogen peroxide, and acid significantly increased the resistance of the pathogen to heat (157).

VIII. HEAT RESISTANCE OF SPORES OF FOODBORNE PATHOGENS

The heat resistance of spores of *C. botulinum* has been studied more thoroughly than those of most other spore-formers. This is because the pathogen produces a potent neurotoxin that can cause various symptoms of paralysis and is thus the most hazardous spore-forming foodborne pathogen. *C. botulinum* type A and B (proteolytic) strains produce highly heat-resistant spores and are of primary importance to food safety. These spores are targeted for destruction to assure microbiological safety of low-acid foods. The canning industry historically adopted a D-value at 121°C of 0.2 minute (12 log reduction) as a standard for designing a required thermal process for an adequate degree of protection against

C. botulinum. In contrast, the heat resistance of *C. botulinum* type E and nonproteolytic strains of type B is comparatively low and has been thoroughly investigated in aqueous media as well as in a variety of foods (Table 3). The greatest food safety concerns in foods preserved by pasteurization and refrigeration is the survival of nonproteolytic *C. botulinum*; spores of those strains that survive the thermal process would pose a botulism hazard even under proper refrigeration temperatures if a secondary barrier, e.g., low pH, low a_w, is not present. However, the thermal process for cook/chill foods is not designed for the destruction of proteolytic strains of *C. botulinum* because these strains do not grow at or below 10°C.

Gaze and Brown (158) assessed the heat resistance over the temperature range 70–92°C of nonproteolytic *C. botulinum* type B and E spores heated in homogenates of cod (pH 6.8) and carrot (pH 5.7). It was calculated that a heat treatment at 90°C for 7 minutes or equivalent time-temperature combination would be sufficient to achieve a 6-D process for the most heat-resistant nonproteolytic *C. botulinum* spores. Juneja et al. (159) reported that turkey should be heated to an internal temperature of 80°C for at least 27.1 minutes to achieve a 6-D process for nonproteolytic *C. botulinum* spores.

Composition of the sporulation medium and the temperature used for spore preparation influence the spore heat resistance. The highest observed heat resistance corresponds to the *C. perfringens* spores obtained in the pH range 7.0–9.0 (160). Spores of *B. cereus* produced in the pH range 6.5–8.3 showed a decline in D-values at 100°C by about 65% per pH unit (161). While sporulation temperature can influence heat resistance, there is no general trend. According to some authors, spores produced at higher temperatures have greater heat resistance (26,38,162). However, some investigators have found the opposite effect

Table 3 Heat Resistance of *C. botulinum* Type E and Nonproteolytic Type B Spores in Buffer and a Variety of Foods

Strains	Menstruum	Temperature (°C)	D-value (min)	Ref.
Type E	Phosphate buffer	80	1.03–4.35	159
Type E	Phosphate buffer	82.2	0.37–0.52	220
Type E	Oyster homogenate	80	0.78	221
Type E	Mehnaden surimi	82.2	1.22	222
Type E	Turkey	70	51.89	159
Type E	Turkey	85	1.18	159
Type B	Phosphate buffer	82.2	1.49–73.6	220
Type B	Phosphate buffer	80	3.22–4.31	159
Type B	Turkey	75	32.53	159
Type B	Turkey	90	0.80	159

(163,164). It has also been shown that heat resistance increases with sporulation temperature up to a maximum, decreasing thereafter (165). Sala et al. (162) reported that the sporulation temperature induces important changes in the influence of the pH of the heating menstruum on the heat resistance of *Bacillus subtilis*. Spores sporulated at 32°C were less heat resistant at pH 4 than at pH 7 regardless of the temperature of treatment. However, the effect of acidic pH on heat resistance decreased as heating temperature increased for spores sporulated at 52°C. While the z-value of a spore suspension sporulated at 32°C did not change irrespective of the pH of heating menstruum, the z-value of suspension sporulated at 52°C increased with acidification. This potential for increase in z-value is a hazard that should be taken into account in hot climates, especially when designing sterilization processes for acidified foods.

Composition and pH of the heating menstruum and the presence of any antibacterial agents during the heating process will substantially affect the heat resistance of bacterial spores. Juneja et al. (159) reported a concomitant increase in heat resistance of nonproteolytic *C. botulinum* spores in turkey slurry as compared to phosphate buffer. While D-value at 80°C for type E spores in buffer was 4.35 minutes, turkey slurry offered protection, and the D-value was increased to 13.37 minutes. Similarly, D-values for the nonproteolytic type B strain in buffer and turkey were 4.31 and 15.21 minutes, respectively. Generally, thermal resistance of spores decreases as the pH of the heating medium is reduced and increases with decreasing a_w. Using *C. botulinum* type A and B strains, Odlaug and Pflug (166) reported D-values in tomato juice (pH 4.2) that were three times lower than in phosphate buffer (pH 7). Xezones and Hutchings (167) reported D-values at 115.5°C of 0.128, 2.6, 4.91, and 5.15 minutes for *C. botulinum* 62A spores suspended in spaghetti sauce at pH 4, 5, 6, and 7, respectively. For *C. botulinum* type E spores, D-values at 110°C of less than 0.1 second at a high a_w were increased by more than 100,000 times as the a_w was lowered to 0.2–0.3 (168). It has also been reported that the influence of a_w on the heat resistance is more pronounced for species with more heat sensitivity (169). In addition, researchers have reported that the effect of a_w of the heating menstruum depended on the treatment temperature (170). Mazas et al. (171) reported that the effects of a_w on the D-values of *B. cereus* depended on the nature of the compound used to control a_w, the strain tested, and the heating temperature. In their study, the protective effect of NaCl against heat on the *B. cereus* spores was higher than that of LiCl at the same a_w values, and that of sucrose was higher than that of glycerol.

Fat and proteins protect spores against heat lethality (172,173). Spores are more resistant to heat when suspended in oils than in buffer. This resistance is ascribed to the low water activity of oils and to their content of free fatty acids. Molin and Snygg (174) reported a D-value at 95°C of 13 minutes for *B. cereus*

spores suspended in phosphate buffer and D-values at 121°C of 17.5–30 minutes for the same spores heated in olive and soybean oils. Survivor curves of the *B. subtilis* spores suspended in olive oil were concave upward with a characteristic tailing (175).

Nisin has been shown to reduce the thermal processing requirements in several food products by enhancing the thermal inactivation of bacterial spores. In a study by Wandling et al. (176), when heat resistance of *B. cereus* was assessed in skim milk supplemented with various concentrations of nisin, the D-values at 97°C were 7.0, 4.8, and 4.7 minutes for the control and 2000 and 4000 IU/mL nisin treatments, respectively. In the same study, the apparent D-values of *B. stearothermophilus* at 130°C were reduced by 13 and 21% because of the presence of 2000 and 4000 IU of nisin/mL, respectively.

Determination of spore survival after exposure to heat is characterized by the ability of the injured spores (which are more nutritionally demanding than unheated spores) to germinate, outgrow, and form colonies on the recovery media. In fact, any factor influencing repair mechanisms might also influence the measured D-value. In well-documented reviews on the recovery of injured spores, Roberts (177) and Adams (178) outlined the importance of the recovery media for injured spores and classified the additives that can increase recovery according to their mechanisms of action. Recovery conditions including the composition and pH of the medium, nature of acidulants, the presence of inhibitors, and temperature and time of incubation can significantly affect survival counts and, consequently, the values obtained for the parameters used to characterize heat resistance (179,180). Feeherry et al. (181) observed a decrease in D-values at 121°C when the recovery medium was acidified with HCl. Fernandez et al. (182) described a higher inhibition of citric acid compared with glucono-δ-lactone on spore recovery. Potassium sorbate at concentrations as low as 0.025% and sodium benzoate at 0.1% were very effective inhibitory agents for heat-injured spores of *B. stearothermophilus* (183). It is usually considered that the most adequate temperature for the recovery of heat-treated spores is lower than the optimal for unheated spores (180). Lower incubation temperatures lead to higher D-values and longer shoulders (184).

There is sufficient evidence to document that the addition of lysozyme to the recovery medium resuscitates and increases the recovery of the heat-injured spores, thereby increasing the measured or apparent heat resistance (159,185, 189). In a study by Sebald et al. (189), when *C. botulinum* type E spores were heated in phosphate buffer for 10 minutes at 80°C, surviving spores were able to form colonies on a medium containing lysozyme, but not in its absence. Alderton et al. (185) reported an estimated D-value of 1.30 minutes when type E spores were heated in phosphate buffer at 79.5°C and recovered on a medium without lysozyme. In the same study, when the recovery medium contained lyso-

zyme, the heating temperature had to be raised to obtain measurable spore destruction; the D-values were 13.50 and 3.80 minutes at 90.5 and 93.3°C, respectively.

In a study by Peck et al. (187), when spores of nonproteolytic *C. botulinum* type B strains were heated at 85°C and survivors were enumerated on a highly nutritive medium, a 5 decimal kill in less than 2 minutes was observed. However, enumeration of survivors on a medium supplemented with lysozyme showed that heating at 85°C for 5 minutes resulted in only an estimated 2.6 decimal kill of spores; also, biphasic survivor curves indicating heat-sensitive and heat-resistant population subfractions were observed. Treatment with alkaline thioglycollate resulted in heated spores being permeable to lysozyme, and a biphasic heat-inactivation curve was converted to a logarithmic curve, the slopes of which were similar to the second part of the biphasic curves (190). This treatment is known to rupture disulfide bonds in the spore coats, thereby making the spore coat permeable to lysozyme. These findings have implications for assessing heat treatments necessary to reduce risk of nonproteolytic *C. botulinum* survival and growth in cook/chill foods.

Duncan et al. (191) suggested that the heat alteration of the spore results in inactivation of the cortex lytic enzyme system, i.e., the system responsible for cortical degradation during germination. Lysozyme in the plating medium can replace the thermally inactivated spore germination enzymes (188). Lysozyme permeates the spore coat and degrades the cortex, leading to core hydration and, consequently, spore germination (13). The lysozyme levels in foods of plant and animal origin are 1.8–27.6 and 20–160 μg/g, respectively. Since lysozyme is heat stable and is present in a variety of foods, the influence of this enzyme in the recovery of heat-damaged spores warrants further investigation to determine its effect on the efficacy of recommended heat processes.

Simulating the conditions in minimally processed refrigerated foods, Juneja and Eblen (192) recovered heated nonproteolytic *C. botulinum* spores on both reinforced clostridial medium (RCM) with lysozyme and on RCM with lysozyme and the same salt levels as the heating menstruum. This approach of heating in a food substrate and recovery under the same conditions is the closest to the real situation in food. When the recovery medium contained no salt, D-values in turkey slurry containing 1% salt were 42.1, 17.1, 7.8, and 1.1 minutes at 75, 80, 85, and 90°C, respectively. The D-values were 27.4, 13.2, 5.0, and 0.8 minutes at 75, 80, 85, and 90°C, respectively, when both the turkey slurry and the recovery medium contained 1% salt. Increasing levels (2–3%, w/v) of salt in turkey slurry resulted in parallel decrease in the D-values obtained from the recovery of spores on the media containing the same levels of salt as the heating menstruum. Hutton et al. (193) reported the presence of salt at 2% levels in the modified PA3679 agar decreased the *C. botulinum* spores D-value by 20–40% irrespective of the pH of the heating menstruum. However, the protective effect of salt leading to

increased D-values is also well known. Inclusion of salt in heating menstruum results in increased heat resistance due to reduced water activity leading to spore dehydration. Juneja and Eblen (192) indicated that the decrease in D-values obtained from the recovery of heat-damaged spores on the media with added salt was a consequence of the inability of heat-injured spores to recover in the presence of salt. The heat-injured spores are sensitive to salt in the recovery medium. These data should assist food processors in designing reduced thermal processes that ensure safety against nonproteolytic *C. botulinum* type B spores in cook/chill foods while maintaining desirable organoleptic attributes of foods.

IX. HEAT-INACTIVATION KINETICS PREDICTIVE MODELS

The effectiveness of the individual effects of heat treatment, pH, salt, etc., with regard to pathogen inactivation is maximized by conducting multiple factorial experiments in which the effects and interactions of these parameters in foods are assessed in lowering the heat resistance of foodborne pathogens. Subsequently, inactivation kinetics or thermal death models are developed which predict the target pathogen's survival within a specific range of food formulation variables. These models can help either to establish an appropriate heat treatment or to understand and determine the extent to which existing/traditional thermal processes could be modified for a variety of cooked foods. The models can contribute to more effective evaluation and assessment of the impact of changes in food formulations that could affect their microbiological safety or the heat lethality of pathogens. These predictive models enable food processors and regulatory agencies to ensure critical food safety margins by predicting the combined effects of multiple food formulation variables. The food processors are able to design appropriate processing times and temperatures for the production of safe food with extended shelf life without substantially adversely affecting the sensory quality of the product. However, it is of critical importance that the D-values predicted by the models first be validated with heat resistance data obtained by actual experiments in specific foods before the predicted values can be used to design thermal processes for the production of a safe food.

Juneja et al. (194,195) and Juneja and Eblen (196) employed a fractional factorial design to assess and quantify the effects and interactions of temperature, pH, salt, and phosphate levels and found that the thermal inactivation of nonproteolytic *C. botulinum* spores, *E. coli* O157:H7, and *L. monocytogenes* was dependent on all four factors. Thermal resistance of spores or vegetative cells can be lowered by combining these intrinsic factors. The following multiple regression equations, developed in these studies, predict D-values of nonproteolytic *C. botulinum* spores for any combinations of temperature (70–90°C), salt (NaCl; 0.0–3.0%), sodium pyrophosphate (0.0–0.3%), and pH (5.0–6.5); *E. coli* O157:H7,

for combinations of heating temperature (55–62.5°C), salt (0.0–6.0%, w/v), sodium pyrophosphate (0.0–0.3%, w/v), and pH (4.0–6.5); and *L. monocytogenes*, for combinations of heating temperature (55–6.5°C), salt (0.0–6.0%, w/v), sodium pyrophosphate (0.0–0.3%, w/v), and pH (4.0–8.0). The predicted D-values are for changes in the parameter values in the range tested from any combination of four environmental factors.

1. Nonproteolytic *C. botulinum* spores:

$$\begin{aligned}\text{Log}_e \text{ D-value} = {} & -9.9161 + 0.6159(\text{temp}) - 2.8600(\text{pH}) \\ & - 0.2190(\text{salt}) + 2.7424(\text{phos}) \\ & + 0.0240(\text{temp})(\text{pH}) - 0.0041(\text{temp})(\text{salt}) \\ & - 0.0611(\text{temp})(\text{phos}) + 0.0443(\text{pH})(\text{salt}) \\ & + 0.2937(\text{pH})(\text{phos}) - 0.2705(\text{salt})(\text{phos}) \\ & - 0.0053(\text{temp})^2 + 0.1074(\text{pH})^2 \\ & + 0.0564(\text{salt})^2 - 2.7678(\text{phos})^2\end{aligned}$$

2. *E. coli* O157:H7:

$$\begin{aligned}\text{Log}_e \text{ D-value} = {} & -43.0646 + 1.4868(\text{temp}) + 3.5737(\text{pH}) \\ & - 0.1341(\text{salt}) - 8.6391(\text{phos}) - 0.0419(\text{temp})(\text{pH}) \\ & + 0.0103(\text{temp})(\text{salt}) + 0.1512(\text{temp})(\text{phos}) \\ & - 0.0544(\text{pH})(\text{salt}) + 0.2253(\text{pH})(\text{phos}) \\ & - 0.2682(\text{salt})(\text{phos}) - 0.0137(\text{temp})^2 \\ & - 0.0799(\text{pH})^2 - 0.0101(\text{salt})^2 - 6.4356(\text{phos})^2\end{aligned}$$

3. *L. monocytogenes*:

$$\begin{aligned}\text{Log}_e \text{ D-value} = {} & -61.4964 + 2.3019(\text{temp}) + 1.2236(\text{pH}) \\ & + 0.7728(\text{salt}) + 1.0477(\text{phos}) \\ & - 0.0102(\text{temp})(\text{pH}) - 0.0085(\text{temp})(\text{salt}) \\ & - 0.0566(\text{temp})(\text{phos}) - 0.0210(\text{pH})(\text{salt}) \\ & - 0.4160(\text{pH})(\text{phos}) + 0.1861(\text{salt})(\text{phos}) \\ & - 0.0217(\text{temp})^2 - 0.0273(\text{pH})^2 \\ & - 0.0213(\text{salt})^2 - 13.1605(\text{phos})^2\end{aligned}$$

The authors developed confidence intervals (95%) to allow microbiologists to predict the variation in the heat resistance of the pathogens. Representative observed and predicted D-values of nonproteolytic *C. botulinum* in ground turkey,

Table 4 Observed and Predicted D-Values at 70–90°C of Nonproteolytic *Clostridium botulinum* in ground turkey, *E. coli* O157:H7, and *L. monocytogenes* in beef gravy

Temperature (°C)	pH	% NaCl	% Phosphate	D-value observed (min)	D-value predicted[a] (min)
Nonproteolytic *Clostridium botulinum*					
70	6.50	0.0	0.00	57.7	66.0
70	6.50	1.5	0.15	40.1	46.5
75	6.25	1.0	0.10	39.1	42.3
75	6.25	1.0	0.20	32.9	38.6
90	5.00	0.0	0.00	5.0	6.3
90	5.00	1.5	0.15	3.1	4.8
E. coli O157:H7					
55	4	0.0	0.0	2.8	4.1
55	4	0.0	0.30	1.9	2.7
55	4	6.0	0.30	3.5	4.3
60	4	3.0	0.15	2.1	2.2
60	6	3.0	0.30	1.8	2.1
L. monocytogenes					
55	4	0.0	0.0	5.35	9.03
55	4	6.0	0.0	12.49	15.74
57.5	5	4.5	0.10	6.92	8.05
57.5	5	4.5	0.20	10.61	8.45

[a] Predicted D-values are the 95% upper confidence limits.
Source: Refs. 194–196.

E. coli O157:H7, and *L. monocytogenes* in beef gravy are provided in Table 4. Predicted D-values from the model compared well with the observed thermal death values. Thus, the model provides a valid description of the data used to generate it. Examples of secondary models used to mathematically describe the inactivation rates of microorganisms are provided in Table 5.

Periago et al. (197) used a factorial experimental design to develop a model that describes the combined effect of NaCl (S; 0.5–3%) and pH 5.75–6.7 (in the heating menstruum and recovery medium) on the apparent heat resistance of *B. stearothermophilus* spores suspended in mushroom extract, over the temperature (T) range 115–125°C. Coefficients obtained for the second order polynomial were:

$$\ln(y) = -93.136 + 0.7413 \times T + 20.0194 \times pH + 0.6067 \times S + 0.022153 \times T \times pH - 0.011486 \times T \times S + 0.1033 \times pH \times S - 0.0048545 \times T^2 - 1.7085 \times pH^2 - 0.09131 \times S^2$$

Table 5 Examples of the Secondary Models Used to Describe Inactivation Rates of Microorganisms

Models (Ref.)	Mathematical description (equation)
Arrhenius model[a] (223)	$k = A \exp[(-E_a/R)/T]$
Model[b] z-value (223)	$D = D_r 10^{-(T-T_r)/z}$
	$Z = 2 \cdot 303\ RTT_r/Ea$
Linear-Arrhenius model[c] (224)	$\ln k = C_0 + C_1/T + C_2\ pH + C_3\ pH^2$
A quadratic response surface model represented a polynomial[d] (194–196)	$\ln(y) = c_1 + c_2T + c_3\ pH + c_4S \ldots c_8T^2 + c_9\ pH^2 + c_{10}S^2$
Extension of the Eyrings model[e] (115)	$k = k_0 + \kappa \frac{K_b T}{h} \cdot \left(\exp\left[\frac{\Delta G^*_H}{RT}\right] \cdot [H^+]^{n_H} + \exp\left[\frac{\Delta G^*_{OH}}{RT}\right] \cdot [OH^-]^{n_{OH}} \right)$

[a] A is the rate constant at infinite temperature, Ea is the activation energy of inactivation, R is the gas-low constant; T in degrees Kelvin.

[b] D_r is the D-value at the reference temperature Tr.

[c] $C_0 - C_3$ are fit parameters/coefficients for the particular food-bacterial system.

[d] ln(y) is the natural logarithm of the dependent variable of the model, the D-value, T is the heating temperature, pH is the pH value of the substrate, S is the NaCl concentration and $C_1 - C_{10}$ are the coefficients to be estimated.

[e] k_o, κ, k_b, h, n_H, n_{OH}, ΔG^*_H and ΔG^*_{OH} as defined by Reichart (115).

The authors observed that both pH and NaCl had a considerable influence on *B. stearothermophilus* D-values. For example, at 115°C, when spores were heated and recovered at pH 6.7 with 0.5% NaCl, the D-value obtained was 9.6 minutes, whereas at the same heating temperature, spores heated and recovered at pH 6 with 3% NaCl had a D-value of 3.3 minutes. This represents almost a 90% reduction in thermal resistance.

A log-logistic model has been applied successfully to quantify: the effects of temperature on the thermal inactivation of *L. monocytogenes* (8), the effect of heating rate on the inactivation of *L. monocytogenes* (198), the effect of pH and temperature on the survival of *Yersinia enterocolitica* (199), the effects of temperatures below 121°C on the inactivation of *C. botulinum* 213B (200), and the effect of temperature on the inactivation of *S.* Typhimurium (201).

The modified Gompertz equation was effectively used to model *L. monocytogenes* inactivation in a formulated infant product with varying temperature (50, 55, 65°C), pH (5.0, 6.0, 7.0), and NaCl concentration (0, 2, 4%) (202). More recently, Blackburn et al. (203) used a log-logistic function to develop a three-factor thermal inactivation models for *S.* Enteritidis and *E. coli* O157H7 as affected by temperature (54.5–64.5°C), pH (4.2–9.6 adjusted using HCl or NaOH), and NaCl concentration (0.5–8.5% w/w). In this study, 84% of *S.* Enteritidis and

83% of *E. coli* O157H7 survival curves represented a linear logarithmic death, with the remaining curves demonstrating shoulder and tailing regions. Chhabra et al. (204) developed a predictive model using a modified Gompertz equation to estimate *L. monocytogenes* death in a formulated and homogenized milk system as affected by milkfat (0–5%), pH (5.0–7.0), and processing temperature (55–65°C). The shoulder region of the survival curve was affected by pH; however, the maximum slope was affected by temperature, milkfat, and the interaction of temperature and milkfat, thereby suggesting that both temperature and milkfat play a role in the rate of *L. monocytogenes* inactivation during thermal processing. Regardless of which equation is used to represent data, predictive model is a useful initial tool to estimate pathogen inactivation and gives an increased understanding of how various conditions affect the death of pathogens.

Mafart and Leguerinel (205) emphasized that the use of D- and z-values is not sufficient and that the ratio of spore recovery after incubation should be considered in calculations used in thermal processing of foods. The authors derived a model describing the recovery of injured spores as a function of both the heat treatment intensity and the recovery conditions, i.e., incubation temperature, pH, and sodium chloride content. When heated spores are recovered under unfavorable conditions, the ratio of cell recovery and apparent D-value are reduced (205).

X. COMBINATION TREATMENTS

Severe heat treatments can impair the organoleptic properties and nutritional values of foods. To avoid the undesirable effects of heat, one approach is to use heat in combination with other already known preservation techniques. The use of combination preservation treatments incorporating mild heat can result in enhanced preservative action by having an additive or synergistic effect on microbial inactivation—particularly in foods with a high water content—and/or reduce the severity of one or all the treatments. A few examples include:

1. The lethal effect of heat is enhanced if bacterial cells have undergone ultrasound treatment (206). The combination treatment of heat and ultrasound is termed "thermosonication" (207).
2. For spores, a combination of high pressure and high temperature is necessary for inactivation. Under high pressure, bacterial spores germinate to vegetative cells and are then inactivated by the heat.
3. An irradiation dose of 5 kGy is sufficient to sensitize clostridial spores to subsequent heating (208). Prior exposure to ionizing radiation stimulates spore germination, rendering the spores sensitive to subsequent heat treatment. Irradiation can also sensitize vegetative cells to subse-

quent heating. Thayer et al. (209) reported that irradiation at a dose of 0.9 kGy caused heat sensitization of *S.* Typhimurium in mechanically deboned chicken. A dose of 0.8 kGy was sufficient to increase the heat sensitivity of *L. monocytogenes* in roast beef and gravy (210). Since the principal target of ionizing radiation is DNA, vegetative cells treated first by ionizing radiation experience damage to their DNA, and then subsequent heat treatment damages enzymes necessary for DNA repair.

4. Efficacy of the lethal effect of heat on microorganisms is increased if subsequently exposed to organic acids. This is a consequence of prior heating causing damage to the cell membrane, making it easier for weak acids to penetrate into the cytoplasm.

XI. CONCLUSIONS AND FUTURE OUTLOOK

The use of heat for the inactivation of microorganisms is the most common process in use in food preservation today. Heat treatment designed to achieve a specific lethality for foodborne pathogens is one of the fundamentally important strategies used to assure the microbiological safety of thermally processed foods. Heat resistance of microorganisms can vary depending on the species and strain of bacteria, food composition, physiological stage of microbial cells or spores, and recovery conditions (type of media, temperature, atmosphere, and time of incubation) for the detection of survivors. Food characteristics leading to increased heat resistance of an organism include water activity and the presence of carbohydrates, lipids, proteins, salt, etc. Heat resistance of spores is attributed primarily to thermal adaptation, mineralization, and dehydration. Alterations in membrane fatty acid profile results in an altered response to subsequent heat treatment. Potential targets of heat damage include nucleic acid, proteins and enzymes, and cellular membranes.

Quantitative knowledge of the factors in food systems that interact and influence the inactivation kinetics are required to accurately estimate how a particular pathogen is likely to behave in a specific food. There is a need for a better understanding of how the interaction among preservation variables can be used for predicting safety of minimally processed, ready-to-eat foods. The effects and interactions of temperature, pH, sodium chloride content, and sodium pyrophosphate concentration are among the variables that researchers have considered when attempting to assess the heat-inactivation kinetics of foodborne pathogens. Incorporation of these multiple barriers increased the sensitivity of cells/spores to heat, thereby reducing heat requirements and ensuring the safety of ready-to-eat food products.

The future of thermal death determination of bacteria will likely rely on predictive thermal inactivation kinetics modeling. Complex multifactorial experi-

ments and analysis to quantify the effects and interactions of additional intrinsic and extrinsic factors and development of "enhanced" predictive models are warranted to ensure the microbiological safety of thermally processed foods. In view of the continued interest in minimally processed foods, it would be logical to define a specific lethality at low temperatures. It would be useful to determine the possible effects of injury to vegetative cells and spores that may result from mild heat treatments and the factors in foods that influence the recovery of cells/spores heated at these low temperatures. In conclusion, future research should focus on conducting dynamic pasteurization (low-temperature, long-time cooking) studies to assess the integrated lethality of cooking and develop integrated predictive models for pathogens for the thermal inactivation, injury, repair, and behavior in ready-to-eat meats including those packaged in modified atmospheres.

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